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# The Bloom's Syndrome Helicase Interacts Directly with the Human DNA Mismatch Repair Protein hMSH6

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**Bloom's syndrome (BS) is a rare genetic disorder characterised by genome instability and cancer susceptibility. BLM, the BS gene product, belongs to the highly-conserved RecQ family of DNA helicases. Although the exact function of BLM in human cells remains to be defined, it seems likely that BLM eliminates some form of homologous recombination (HR) intermediate that arises during DNA replication. Similarly, the mismatch repair (MMR) system also plays a crucial role in the maintenance of genomic stability, by correcting DNA errors generated during DNA replication. Recent evidence implicates components of the MMR system also in HR repair. We now show that hMSH6, a component of the heterodimeric mismatch recognition complex hMSH2/hMSH6 (hMutSα), interacts with the BLM protein both *in vivo* and *in vitro*. In agreement with these findings, BLM and hMSH6 colocalise to discrete nuclear foci following exposure of the cells to ionising radiation. However, the purified recombinant MutSα complex does not affect the helicase activity of BLM *in vitro*. As BLM has previously been shown to interact with the hMLH1 component of the hMLH1/hPMS2 (hMutLα) heterodimeric MMR complex, our present findings further strengthen the link between BLM and processes involving correction of DNA mismatches, such as in the regulation of the fidelity of homologous recombination events.**

*Key words:* BLM helicase / Bloom's syndrome (BS) / Genome stability / Homologous recombination (HR) / Mismatch repair (MMR) / Protein-protein interactions.

## Introduction

Bloom's syndrome (BS) is a rare autosomal recessive disorder characterised by growth retardation, immunodeficiency, sun sensitivity, genomic instability, and a strong predisposition to many types of cancer (German, 1993). BS is caused by mutations in the *BLM* gene, which encodes a DNA helicase of the RecQ family (Ellis *et al.*, 1995). Other members of this highly-conserved group of proteins include the *E. coli* RecQ (Nakayama *et al.*, 1985), *S. cerevisiae* Sgs1p (Gangloff *et al.*, 1994), *S. pombe* Rqh1p (Stewart *et al.*, 1997), and four additional human homologues, *RECQL* (Puranam and Blackshear, 1994); *WRN*, the gene mutated in the premature aging disorder Werner's syndrome (Yu *et al.*, 1996); *RECQL4*, the gene mutated in Rothmund-Thomson syndrome (Kitao *et al.*, 1999); and *RECQL5* (Kitao *et al.*, 1998). The most obvious abnormalities observed in cells derived from BS individuals are the highly increased frequency of sister-chromatid exchanges (SCEs), chromatid breaks and gaps, and rearranged chromosomes (German *et al.*, 1974). The *BLM* gene encodes a nuclear protein consisting of 1417 amino acids (Ellis *et al.*, 1995) that possesses a 3'→5' DNA helicase activity on a variety of different DNA substrates (Karow *et al.*, 1997; Sun *et al.*, 1998; Mohaghegh *et al.*, 2001). The *BLM* mutations in BS individuals disrupt the helicase activity of BLM, often resulting in the expression of an mRNA that is unstable. This suggests that BS results from the absence of BLM helicase activity/protein, and that BLM is not an essential protein in humans (Ellis *et al.*, 1995). Consistent with this hypothesis, a viable mouse model of BS has been developed through gene targeting, in which *BLM* mRNA and protein expression are absent or undetectable (Luo *et al.*, 2000). Immunofluorescence studies revealed that BLM is present in nuclear bodies containing the promyelocytic leukemia protein (PML) (Ishov *et al.*, 1999; Sanz *et al.*, 2000; Yankiwski *et al.*, 2000; Bischof *et al.*, 2001), in diffuse patches in the nucleolus (Yankiwski *et al.*, 2000), and at telomeres in telomerase-negative tumor cells (Stavropoulos *et al.*, 2002).

Although the precise function of BLM in human cells remains poorly defined, considerable evidence suggests that the BLM protein plays a role in homologous recombination (HR), probably by participating in the process of HR-mediated replication restart at sites of stalled replication forks. Genetic studies utilising the *S. cerevisiae SGS1* gene have played a crucial role in furthering our understanding of the function(s) of RecQ helicases such as BLM in the process of HR. Deletion of *SGS1* leads to an increase in the frequency of several types of DNA recom-

bination, as well as a breakdown in the fidelity of chromosome segregation during mitosis and meiosis (Gangloff *et al.*, 1994; Watt *et al.*, 1995, 1996). In addition, interspecies cross-functionality between *BLM* and *SGS1* has been indicated by the observation that *BLM* is capable of partially suppressing the hyper-recombination phenotype of *sgs1* mutants and restoring the slow growth phenotype of a *top3 sgs1* double mutant (Yamagata *et al.*, 1998). Further evidence for a role of BLM in HR comes from the observation that BLM directly interacts with RAD51, and co-localises with it in the nucleus of cells exposed to DNA damaging agents (Bischof *et al.*, 2001; Wu *et al.*, 2001). Furthermore, the enzymatic activity of the BLM helicase is also consistent with a role in HR: BLM can disrupt synthetic D-loop structures and catalyse branch migration of Holliday junctions (HJ) and synthetic four-way junctions (Karow *et al.*, 2000; van Brabant *et al.*, 2000; Mohaghegh *et al.*, 2001), which arise as intermediates during HR, and may occur spontaneously during DNA replication and repair (Karow *et al.*, 2000). BLM has recently been shown to interact with p53 (Garkavtsev *et al.*, 2001; Wang *et al.*, 2001). This interaction attenuates the ability of BLM to unwind synthetic HJ *in vitro* (Yang *et al.*, 2002) and is necessary to transport p53 to sites of stalled DNA replication forks where the two proteins functionally interact to modulate HR (Sengupta *et al.*, 2003). Moreover, the presence of BLM in the two multiprotein complexes BASC (BRCA1-Associated genome Surveillance Complex), together with hMSH2, hMSH6, hMLH1, ATM, the RAD50-MRE11-NBS1 complex and replication factor C (Wang *et al.*, 2000), and BRAFT, containing also five Fanconi anemia complementation group proteins, topoisomerase IIIα and replication protein A (Meetei *et al.*, 2003), suggests a functional link to DNA repair.

The DNA mismatch repair (MMR) system is a conserved pathway involved in the removal of mispaired bases from DNA, which plays an important role in the maintenance of genomic stability in both prokaryotes and eukaryotes (reviewed in Bellacosa, 2001). The *E. coli* MutHLS MMR pathway has been well characterised biochemically and genetically (Modrich and Lahue, 1996), and has served as a paradigm for the yeast and mammalian MMR pathways. A number of homologues of MutS and MutL MMR proteins have been described in yeast and mammalian cells. Base/base mismatches and small insertion/deletion loops are recognised by the hMutSα complex, which is a heterodimer of hMSH2 and hMSH6. hMSH2 also pairs with another MutS homologue, hMSH3, to form a heterodimer known as hMutSß, which is involved in the repair of larger insertion/deletion loops. Following this initial mismatch-recognition step, homologues of the bacterial MutL ATPase, predominantly the hMLH1-hPMS2 (hMutLα) heterodimer in humans, couple mismatch recognition to the appropriate downstream processing steps. Interestingly, defects in some of the MMR proteins lead to an inherited cancer syndrome called hereditary non-polyposis colon cancer (HNPCC). Mutations in two MMR genes, *hMSH2* and *hMLH1*, have typically been associated with HNPCC, while mutations in other MMR genes (*hMSH6, hPMS1*, and *hPMS2*) are rare.

In addition to their role in the repair of replication errors, MMR proteins have been implicated in some aspects of HR (reviewed in Evans and Alani, 2000; Bellacosa, 2001). In mammalian cells, as in other organisms, HR is well established as one of the major pathways for the repair of DNA double-strand breaks. MMR components have been shown to function in HR by suppressing recombination between homeologous sequences (similar, but not identical), a role that appears to be conserved in bacteria, yeast, and mammals (Modrich and Lahue, 1996). The hMutSα complex has also been shown to bind to HJs, suggesting that it may be involved in additional HR processes *in vivo* (Marsischky *et al.*, 1999). Moreover, hMSH2$^{-/-}$ and hMSH6$^{-/-}$ murine embyonic stem cells are promiscuous during recombination between homologous sequences in gene-targeting experiments (de Wind *et al.*, 1995, 1999).

We and others have recently demonstrated that BLM interacts directly with the MMR protein hMLH1 (Langland *et al.*, 2001; Pedrazzi *et al.*, 2001). Since BS cells are not deficient in MMR, it has been proposed that the hMLH1 interaction with BLM may play a role in HR (Langland *et al.*, 2001; Pedrazzi *et al.*, 2001). In this study, we set out to test whether BLM interacts also with other components of the MMR system. We demonstrate here that BLM interacts directly with hMSH6, but not with the hMSH2 component of the MutSα heterodimer. Consistent with this notion is the observation that BLM and hMSH6 co-immunoprecipitate from human nuclear extracts and co-localise to nuclear foci in response to ionising radiation. Taken together, our data provide further evidence for a role of BLM helicase alongside MMR proteins in HR.

## Results

### BLM Directly Interacts with hMSH6 But Not with hMSH2

To examine whether BLM interacts with components of the MMR system in addition to hMLH1, we performed a dot-blot assay where we immobilised increasing amounts of purified recombinant MutSα (Iaccarino *et al.*, 1998), MutLα (positive control) (Raschle *et al.*, 1999), or phage protein D (negative control), onto a nitrocellulose membrane, and incubated the membrane with purified recombinant BLM protein (Karow *et al.*, 1997). The presence of bound BLM protein was detected using an antibody against BLM. As shown in Figure 1A, BLM interacts with the MutSα heterodimer, although to a slightly lesser extent than with the MutLα complex. Knowing that BLM directly interacts with at least one of the components of MutSα complex, we sought to identify whether this inter-

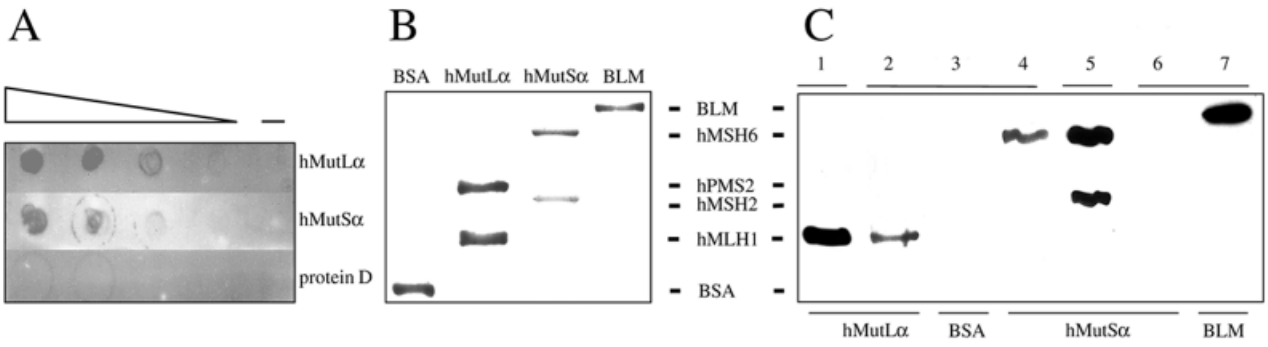


**Fig. 1** BLM Interacts Directly with hMSH6, But Not with hMSH2.
(A) Purified recombinant BLM can bind to immobilised purified recombinant MutSα. The bound BLM was detected using an anti-BLM antibody. (B) Coomassie Blue stained gel showing the purified BSA (1 µg), MutLα (1.5 µg), MutSα (1 µg) and BLM proteins (1 µg) used in (C). (C) Far-Western analysis. 0.5 µg of MutLα (lanes 1, 2), 1 µg of BSA (lane 3), 1 µg of MutSα (lanes 4 – 6) and 0.2 µg of BLM (lane 7) were separated by SDS-PAGE and blotted onto a nitrocellulose membrane. After renaturation, lanes 2 to 4 of the membrane were incubated with purified recombinant BLM (1 µg/ml) and the presence of bound BLM protein was detected by Western analysis using an anti-BLM antibody. Lane 1 was probed with an antibody against hMLH1, lane 5 with antibodies against hMSH2 and hMSH6 and lanes 6 and 7 were probed with an anti-BLM antibody.

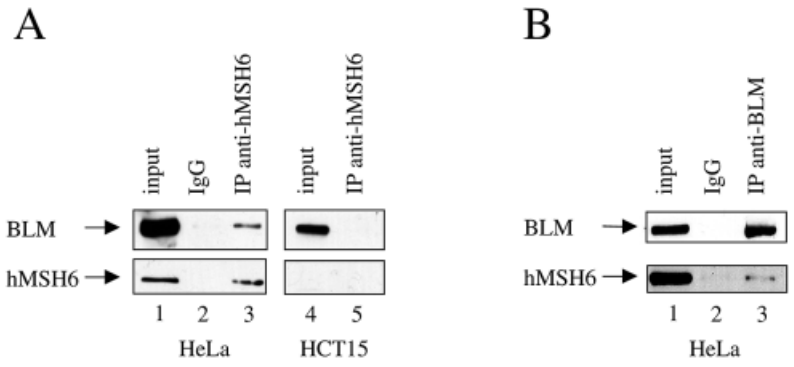


**Fig. 2** BLM and hMSH6 Exist as a Complex in Human Cells.
(A) Co-immunoprecipitation of BLM with hMSH6. BLM could be co-immunoprecipitated with an anti-hMSH6 antibody from 200 µg of HeLa nuclear extract (lane 3), but not with IgG (lane 2). Lane 1 shows the input (20 µg). The proteins were visualised by Western blot analysis with antibodies against BLM (upper) or hMSH6 (lower). (B) Co-immunoprecipitation of hMSH6 with BLM. hMSH6 was immunoprecipitated from 200 µg of HeLa nuclear extract (lane 3) with an anti-BLM antibody but not with IgG (lane 2). The proteins were detected with antibodies against BLM (upper) or hMSH6 (lower).

action was with hMSH2 and/or hMSH6. For this, we performed a Far-Western blot analysis. Figure 1B shows a Coomassie Blue stained gel with the purified recombinant proteins. MutSα was separated into its two components, hMSH2 and hMSH6, by SDS-PAGE and the proteins were then renatured on the membrane after blotting. Subsequently, lanes 2 to 4 (Figure 1C) of the membrane were probed with purified recombinant BLM protein. Western analysis with an anti-BLM antibody (IHIC33) revealed bands at the positions corresponding to hMHS6 (lane 4) and the positive control hMLH1 (lane 2) after incubation with BLM as well as for the BLM input (lane 7). No signal was detected at the positions of the negative controls hPMS2 (lane 2) and BSA (lane3) nor at the position of the hMSH2 protein (lane4). Cross-reactivity of the anti BLM antibody with hMSH6 can be excluded, as it did not recognize the protein that had not been incubated with BLM (lane 6). The amount of BLM bound to hMSH6 strongly increased when MutLα complex was added to the reaction (data not shown), although the mechanism underlying this effect is not known and awaits further investigation.

### BLM and hMHS6 Form a Complex in Human Cells

To confirm the BLM/hMSH6 interaction detected by the Far-Western assay, and to assess whether this interaction can be detected in human cells, we performed co-immunoprecipitation experiments on nuclear extracts from HeLa cells. Using a monoclonal antibody against hMSH6, we were able to specifically co-immunoprecipitate BLM (Figure 2A, lane 3). No BLM was present in the precipitate when a control antibody was used (Figure 2A, lane 2). Similarly, BLM could not be co-immunoprecipitated with an anti-hMSH6 antibody from hMSH6-deficient HCT15 nuclear extracts (Figure 2A, lane 5). Reciprocal co-immunoprecipitation experiments showed that hMSH6 could specifically be precipitated with a poly-

clonal anti-BLM antibody (Figure 2B, lane 3) from HeLa nuclear extracts. We observed no increase in the amount of co-immunoprecipitated BLM/hMSH6 complex upon the addition of native DNA or mismatched DNA (data not shown).

### Mapping of BLM and hMSH6 Interaction Regions

To investigate the region of BLM protein that is responsible for mediating the interaction with hMSH6, different BLM deletion mutants were transcribed and translated *in vitro* (IVTT) and used as radioactive probes to test for their ability to interact with full-length hMSH6 in an *in vitro* binding assay (IBA). As described above, the recombinant MutSα complex was separated by SDS-PAGE and its constituent polypetides were renatured on the membrane after blotting. As a negative control, BSA was included on the membrane. Where possible, based on previous mapping data, the translated BLM fragments were tested for their binding to hMLH1, in order to confirm the correct folding of the IVTT BLM fragments. As shown in Figure 3A, the result of the IBA revealed two separate regions on BLM that are required for binding to hMSH6. While no interaction could be observed with the N-terminal BLM-construct (amino acids 1 – 448), both an internal fragment covering amino acids 340 – 770, and a C-terminal fragment spanning amino acids 904 – 1417 showed binding to hMSH6. Neither of these fragments bound to BSA or hMSH2. We conclude that BLM contains two separate hMSH6-binding regions.

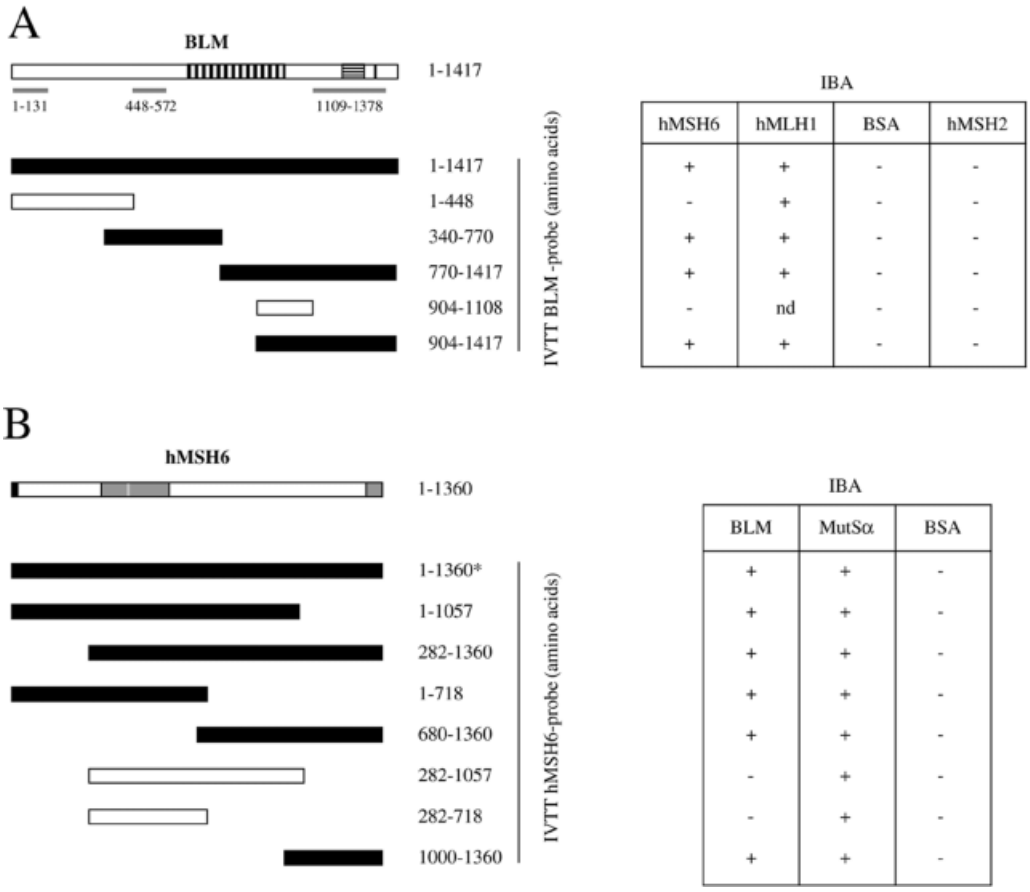


**Fig. 3** Mapping of BLM- and hMSH6-Interacting Regions by an *in vitro* Binding Assay.
(A) Two separate regions on BLM interact with hMSH6. Recombinant MutSα complex (1µg), MutLα (1 µg) and BSA (1 µg) were separated by SDS-PAGE and blotted onto a nitrocellulose membrane. After renaturation, the membranes were incubated with [$^{35}$S]-labelled IVTT BLM fragments. Black bars show fragments that were able to bind to hMSH6, but not to hMSH2 or BSA, white bars represent fragments that do not bind to hMSH6. The Table summarises the obtained results with all tested proteins (+: interaction; –: no interaction; nd: not determined). The sketch of BLM shows the position of the helicase domain (vertically striped, amino acids 649 – 1005), the HRDC domain (horizontally striped, amino acids 1212 – 1292) and the nuclear localisation signals (black bar, amino acids 1334 – 1349). The grey bars below depict the regions mediating the interaction with hMLH1. (B) hMSH6 interacts with BLM *via* two distinct regions. Aliquots of 0, 0.25 and 0.5 µg of purified recombinant BLM protein or MutSα complex (positive control), or 0, 0.5 and 1 µg of BSA (negative control) were spotted onto a nitrocellulose membrane and probed with the indicated IVTT fragments. Black bars represent positive interactions, white bars non-interacting fragments. An overview of all tested interactions is shown in the Table (+: interaction, –: no interaction). The sketch shows the regions of hMSH6 interacting with PCNA (black, amino acids 3 – 22) and hMSH2 (dark grey, amino acids 326 – 575 and 1302 – 1360) as well as the residue important for mismatch binding (light grey, amino acid 432).
*As full-length IVTT hMSH6 was highly prone to degradation, it was co-produced with hMSH2.

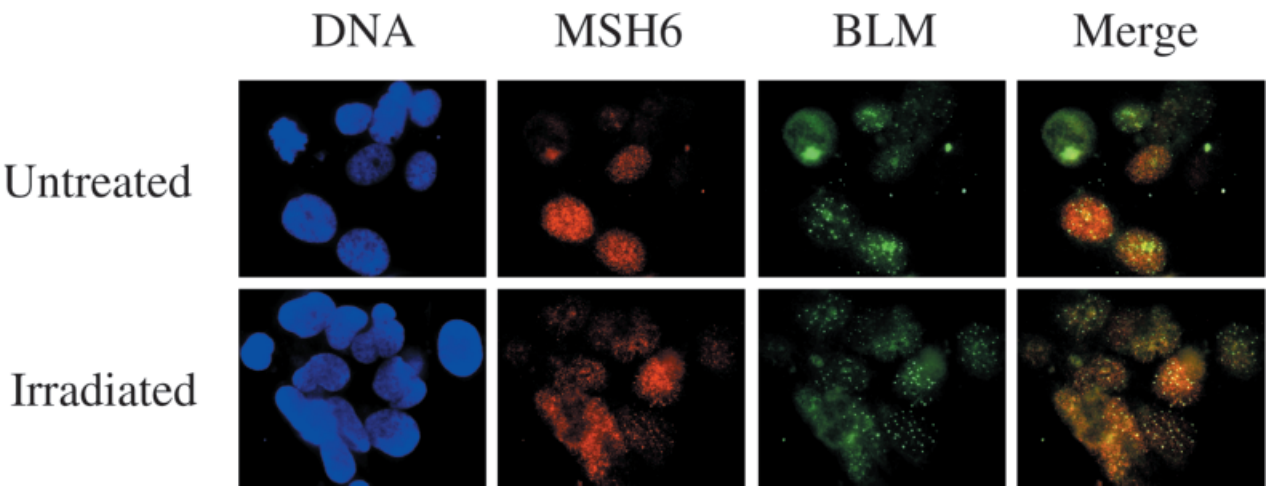


**Fig. 4** BLM Co-Localises with hMSH6 to Nuclear Foci in Irradiated WI-38/VA-13 Cells.
Nuclear foci were detected with the rabbit polyclonal IHIC33 anti-BLM antibody (green) and the mouse monoclonal anti-MSH6 (red) antibody in untreated and irradiated WI-38/VA-13 cells as indicated on the left. The merged image indicates where the red and green foci are coincident, as shown by a yellow colour. Nuclear DNA was stained with Hoechst 33258 dye.

To identify which regions of hMSH6 are involved in binding to BLM, we used a similar approach, in which increasing amounts of purified recombinant BLM protein (and MutSα as a positive control and BSA as a negative control) were dotted onto a nitrocellulose membrane, and probed with [$^{35}$S]-labelled IVTT deletion mutants of hMSH6, or IVTT full-length hMSH6. Due to rapid degradation when produced alone, hMSH6 was co-translated with hMSH2. hMSH2 alone did not interact with BLM (data not shown). Fragments represented by black bars show positive interactions, while white bars show constructs not interacting with BLM (Figure 3B). We conclude therefore, that two BLM-interacting regions are present on hMSH6, one in the N-terminal half (amino acids 1–718) and one at the very C-terminus (amino acids 1000–1360). A more detailed mapping of the N-terminal fragment was not successful as a fragment covering amino acids 1–305 was not able to bind to a positive control (hMSH6-interacting protein), PCNA, possibly due to misfolding, and therefore we were unable to draw any conclusions about a potential interaction with BLM.

### BLM and hMSH6 Co-Localise to Discrete Nuclear Foci in Response to Ionising Radiation

The co-immunoprecipitation of BLM and hMSH6 from human nuclear extracts, as well as the evidence for a direct interaction between purified BLM and hMSH6, is consistent with these proteins forming a complex *in vivo* and *in vitro*. To provide additional evidence for this, we asked whether BLM and hMSH6 co-localise within the nucleus of human cells. Previous studies in HeLa and WI-38/VA-13 human cells have shown that BLM localises to nuclear foci corresponding to PML nuclear bodies (Ishov *et al.*, 1999; Sanz *et al.*, 2000; Yankiwski *et al.*, 2000; Bischof *et al.*, 2001; Wu *et al.*, 2001), as well as to the nucleolus during certain stages of the cell cycle (Yankiwski *et al.*, 2000). However, BLM has also been shown to co-localise with sites of ongoing DNA replication, at least in a subset of late S-phase cells (Wu *et al.*, 2000a). Immunofluorescence studies conducted with MMR-proficient HeLa cells showed that, in cells undergoing DNA replication, hMSH6 co-localises with PCNA to nuclear foci, which most likely represent replication complexes (Kleczkowska *et al.*, 2001). In untreated WI38/VA-13 cells, we found only very rare cases where BLM-containing nuclear foci appeared to co-localise with hMSH6 (Figure 4, upper panel). We therefore asked if the number of co-localising BLM/hMSH6 foci might increase in response to DNA damage. One hour after 10 Gy of γ-irradiation, the proportion of cells containing BLM and hMSH6 co-localising foci increased, although co-localisation (at least to discrete foci) was still a rare event (Figure 4, lower panel).

### The MutSα Complex Does Not Appear to Influence the DNA Helicase Activity of BLM

The observation that BLM and hMSH6 interact directly and can co-localise in nuclear foci following exposure of cells to ionising radiation indicate that the two proteins may be involved in a common cellular pathway. Since the helicase activity of BLM is necessary for the promotion of Holliday junction (HJ) branch migration (Karow *et al.*, 2000; Yang *et al.*, 2002), we investigated whether purified recombinant MutSα complex might modulate the ability of BLM to disrupt a radiolabeled synthetic X-junction substrate, a mimic of the HJ (Figure 5A). Consistent with previous reports (Karow *et al.*, 2000; Yang *et al.*, 2002), the purified recombinant BLM disrupted the X-junction in a dose-dependent manner into primarily two-armed products (the product of branch migration) as well as some one-armed (single-stranded DNA) product generated by the unwinding of the 2-armed species (Figure 5A,

lanes 3–9). In the absence of BLM, purified recombinant MutSα complex did not show any intrinsic helicase activity (Figure 5A, lane 18). To test the effect of MutSα on the ability of BLM to disrupt the X-junction, increasing amounts of BLM (8–25 fmol) were incubated with the X-junction in the presence of a molar excess of purified recombinant MutSα complex (200 fmol). No effect of MutSα on the DNA helicase activity of BLM was observed

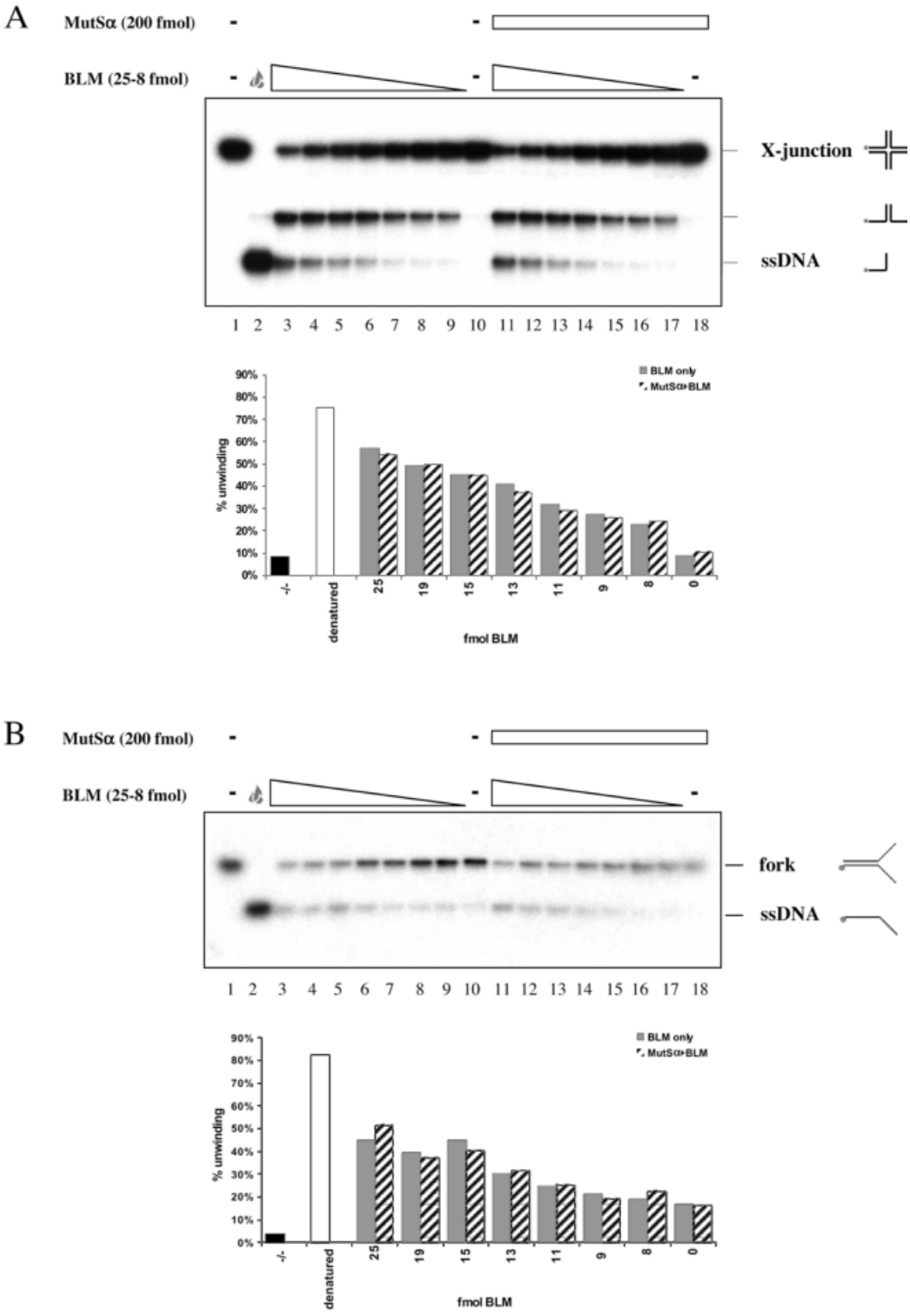


**Fig. 5** Helicase Activity of BLM Is Not Affected by the Purified Recombinant MutSα Complex.
Approximately 0.075 fmol of synthetic X-junction (A) or replication fork (B) substrate were incubated with different concentrations (indicated on the chart) of BLM alone or BLM together with 200 fmol MutSα. The structures of the intact substrate and products of the unwinding reaction are schematically represented on the right of the autoradiograms. The chart shows quantification of the data from the autoradiogram. The flame symbol depicts heat-denatured substrate (empty bar on chart).

under these conditions (Figure 5A, chart), or when increasing amounts of purified recombinant MutSα complex (6.25–400 fmol) were used in reactions where the amount of BLM was kept at a fixed level (11.25 fmol; data not shown).

We next asked if MutSα might influence BLM helicase activity on a different DNA substrate. To that end, a forked DNA structure that mimics a simplified version of a replication fork was incubated with increasing amounts of BLM (8–25 fmol) and a fixed amount of MutSα complex (200 fmol). As shown in Figure 5B (chart), MutSα did not stimulate or inhibit BLM unwinding of a forked DNA structure.

## Discussion

The molecular roles of the BLM gene product in the maintenance of genomic stability in human cells still remain to be defined. In the present study, we have shown that the Bloom's syndrome helicase interacts *in vivo* and *in vitro* with hMSH6, a protein involved in MMR. Hence, BLM makes physical interactions with components (hMSH6 and hMLH1) of the two major MMR heterodimeric complexes, MutSα and MutLα. We have demonstrated that BLM interacts directly with hMSH6 *via* two separate sites comprising the amino acids 340–770 and 904–1417 of BLM. In addition, we have shown that two regions of hMSH6 (amino acids 1–718 and 1000–1360) mediate the interaction with BLM. However, immunofluorescence data suggest that BLM and hMSH6 may only co-localise to a limited extent in response to DNA damage. Further work is required to identify whether co-localisation occurs more dramatically in cells exposed to stresses other than γ-irradiation.

Various experiments performed in the past three years have suggested a likely role for BLM in HR repair through its ability to disrupt synthetic D-loop substrates and/or to promote the ATP-dependent translocation of HJ (Karow *et al.*, 2000; van Brabant *et al.*, 2000; Mohaghegh *et al.*, 2001; Yang *et al.*, 2002), a function that may suppress inappropriate DNA recombination *in vivo.* Consistent with these observations, BLM has been shown to interact with RPA (Brosh *et al.*, 2000), RAD51 (Wu *et al.*, 2001), hMLH1 (Langland *et al.*, 2001; Pedrazzi *et al.*, 2001), and p53 (Wang *et al.*, 2001), four proteins known also to influence HR repair. In the case of the BLM/p53 interaction, Yang *et al.* have demonstrated recently that purified recombinant p53 attenuates the ability of BLM to unwind synthetic HJs *in vitro* (Yang *et al.*, 2002). Thus, a role for BLM as an 'anti-recombinase' in the suppression of genome instability is now suggested. Nevertheless, why should it be necessary for BLM to interact with mismatch repair proteins such as hMSH6 and hMLH1 during this or related HR processes? We suggest that the most likely answer to this question lies in the property of at least some MMR proteins to participate in HR in addition to their role in the post-replicative mismatch repair (reviewed in Bellacosa, 2001). MMR proteins have been shown to be anti-recombinogenic in yeast and bacteria, to be involved in regulation of heteroduplex length in yeast and mice, and to suppress homeologous recombination (reviewed in Modrich and Lahue, 1996; Evans and Alani, 2000; Harfe and Jinks-Robertson, 2000). In addition to these observations, the MutSα complex can bind with high affinity and specificity to HJ (Marsischky *et al.*, 1999), and thus may be involved in the HR repair process *in vivo* by rendering the HJ more accessible to other processing components. These data, combined with evidence that BLM serves to prevent inappropriate HR during DNA replication, lead us to propose that MMR proteins such as hMSH6 and hMLH1 may assist BLM to perform its 'anti-recombinase' function by modulating its branch migration activity leading to the restoration of a functional replication fork structure. In this way, MMR proteins could serve as 'docking sites' to position BLM at sites of HR repair. However, we could not observe any effect of the purified recombinant MutSα complex on the ability of BLM to unwind synthetic HJ in our *in vitro* helicase experiments. Nevertheless, it seems unlikely that BLM and MutSα act alone and therefore any modulation of BLM activity, if it occurs, may require one or more additional components of the MMR or HR machinery.

Clearly, the physical interactions that exist between BLM and hMSH6, and between BLM and hMLH1 (Langland *et al.*, 2001; Pedrazzi *et al.*, 2001), strongly suggest that BLM is intimately linked to some aspect(s) of the MMR process. Given that Bloom's syndrome cells are MMR proficient, BLM is unlikely to function in 'generalised' MMR, but is more likely involved in the regulation of genetic recombination. Further establishing these links by more sophisticated functional assays is a critical next step in the investigation of the functional connections between BLM and MMR proteins during HR. It will also be interesting to determine whether WRN and RECQL4, other members of the RecQ family helicases that are defective in Werner's and Rothmund-Thomson syndromes, respectively, also interact physically and functionally with the MMR proteins. These studies will certainly lead to a deeper understanding of the fundamental roles of the MMR proteins and RecQ helicases in DNA replication and HR repair.

## Materials and Methods

### Hela Nuclear Extracts

Extracts were prepared as described in Perkins *et al.* (1994).

### Construction of Plasmids

The different hMSH6 plasmids for *in vitro* transcription were generated by PCR using the *hMSH6* cDNA as a template, followed by cloning into vectors of the pCite-4 series (Novagen, Madison, USA). For constructs used in interaction site mapping on BLM, see Pedrazzi *et al.* (2001). Sequences of all plasmids and construction schemes are available upon request.

### Co-Immunoprecipitation Experiments

Two-hundred µg of Hela cell nuclear extracts were incubated for one hour at 4 °C in 1× binding buffer (20 mM Tris/HCl pH 7.5, 5 mM $MgCl_2$, 1 mM PMSF, 1 mM glutathione, 0.1 mM dNTPs, 50 µg/ml BSA), supplemented with 10% sucrose and 1× protease inhibitor cocktail, EDTA-free (Roche, Basel, Switzerland) and 0.05 µg/ml polydIdC (Fluka, Buchs, Switzerland) with an effective salt concentration of 80 mM NaCl. Two µg of the mouse monoclonal anti-hMSH6 antibody (MCA 1687; Serotec, Oxford, UK) or a mouse IgG control antibody for the hMSH6 were used in the immunoprecipitation experiments. Alternatively, 4 µg of the polyclonal goat anti-BLM antibody C-18 (Santa Cruz, Santa Cruz, USA) or a goat IgG control antibody were used in BLM immunoprecipitations. In each case, the incubations were continued for two hours. Twenty µl of protein G Dynabeads (Dynal, Hamburg, Germany) were added to the solution and the incubation was continued for a further 1.5 h before the matrix-bead proteins were isolated according to the instructions of the manufacturer. The beads were washed five times with 200 µl of binding buffer (100 mM NaCl for IP with anti-hMSH6 antibody, and 100 mM NaCl plus 150 mM KCl for anti-BLM antibody) before elution with 2× Laemmli buffer. The immunoprecipitated proteins were subjected to Western blot analysis using a polyclonal anti-hMSH6 antibody (Palombo *et al.*, 1995) or the polyclonal anti-BLM IHIC33 antibody (Wu *et al.*, 2000b). Detection was performed using ECL (Amersham Pharmacia Biotech, Uppsala, Sweden) following the manufacturer's instructions.

### Far-Western Analysis

This assay was performed essentially as described previously (Wu *et al.*, 2000b). Briefly, 1 µg of purified hMutSα, 1 µg of BSA and 0.5 µg of purified hMutLα were subjected to SDS-PAGE and transferred to nitrocellulose filters. After renaturation and blocking, the filters were incubated for 60 min in a solution containing BLM (1 µg/ml) in TBS supplemented with 0.25% milk, 0.3% Tween 20, 1 mM DTT and 1 mM PMSF. After extensive washing, conventional Western blotting was performed to detect the presence of BLM (antibody IHIC33). The inputs were visualised with antibodies against hMLH1 (G168-15; Pharmingen, San Diego, USA), hMSH2 (Ab-2; Calbiochem, San Diego, USA), hMSH6 (21F10, Serotec) and BLM (IHIC33). For Figure 1A, MutSα, MutLα and protein D were directly spotted onto the membrane in increasing amounts (0, 0.4, 0.8, 1.6, 3.2 and 6.4 pmoles) and incubated with BLM (3 µg/ml) as described below for the *in vitro* binding assay. Detection of bound BLM protein was as outlined above.

### *In Vitro* Binding Assay

Different amounts of recombinant BLM and BSA were directly spotted onto a nitrocellulose membrane (Micron Separation Inc., Westborough, USA), while hMutSα was subjected to SDS-PAGE prior to transfer to the nitrocellulose membrane followed by renaturation/denaturation steps as described for the Far Western assay. After blocking for one hour at room temperature using TBS supplemented with 5% non-fat milk and 0.5% Tween, the membrane was incubated for three hours at 4 °C with different proteins that were [$^{35}$S]-labelled using the TNT T7 quick-coupled transcription/translation system (Promega, Mannheim, Germany) in 1 ml TBS with 0.5% Tween and 0.1% BSA. Fifty µl (of BLM fragments and full-length hMSH6-hMSH2 complex) or 75 µl (hMSH6 fragments) of the *in vitro* transcription and translation reactions were used for each incubation. After extensive washing with TBS supplemented with 0.5% Tween, the membranes were dried and exposed to a PhosphorImager (Molecular Dynamics, Sunnyvale, USA).

### Indirect Immunofluorescence Analysis

Indirect Immunofluorescence Analysis was performed essentially as described in Wu *et al.* (2000b), with slight modifications. Cells were grown on coverslips and fixed with 4% paraformaldehyde, 250 mM HEPES, pH 7.4 at 4 °C for 20 min, and then permeabilised in 0.1% Triton X-100 in PBSA for 20 min. After washing 5 times in PBSA for 20 min, blocking was carried out at 37 °C for 20 min in 10% foetal bovine serum, 0.1% Triton X-100 in PBSA. The coverslips were incubated for 1 h at 37 °C with the primary antibodies IHIC33, or hMSH6/GTBP (BD Transduction Laboratories, Lexington, USA), which were diluted in the above blocking solution at 1:200, and 1:800, respectively. Five washes in PBSA for 20 min were followed by incubation with anti-mouse Cy3 (Sigma, St. Louis, USA), anti-rabbit fluorescein isothiocyanate (Dako, Glostrup, Denmark) or anti-rabbit AlexaFluor 488 (Molecular Probes, Eugene, USA) secondary antibodies for 1 hour at 37 °C at 1/800, 1/200 and 1/800 dilutions, respectively. Cells were washed five times in PBSA, and the DNA was stained using Hoechst 33258 at 50 ng/ml. Stained slides were mounted in 90% glycerol, 20 mM Tris-HCl, pH 8.0, and 50 µg/ml paraphenylenediamine. Slides were viewed at 100× magnification on a Zeiss Axioskop microscope. Image acquisition and analysis were performed using the AxioVision (Zeiss, Jena, Germany) software, and the images were merged using Adobe Photoshop.

### DNA Helicase Assays

Approximately 0.075 fmol of oligonucleotide-based 4-way junction or forked duplex substrate (Mohaghegh *et al.*, 2001) were incubated with the indicated amounts of BLM and hMutSα proteins in a 10 µl reaction volume at 37 °C for 45 minutes in helicase buffer (33 mM Tris-acetate pH 7.8, 1 mM Mg-acetate, 66 mM K-acetate, 0.1 mg/ml BSA, 1 mM DTT, 1 mM ATP). The reaction was stopped by the addition of 1/10 volume loading buffer (100 mM Tris-HCl, 100 mM EDTA, bromophenol blue and xylene cyanol). The samples were electrophoresed on 10% acrylamide gels in 1× TBE at 25 mA for 1 h at 4 °C. The gels were dried at 80 °C for 30 min and subjected to autoradiography. Quantitative analysis of the rate of unwinding was performed on a Storm 840 PhosphorImager and ImageQuant software (Molecular Dynamics).

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